

FORM PTO-1390
(REV 10-95)

U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

ATTORNEYS DOCKET NO.

TRANSMITTAL LETTER TO THE UNITED STATES
DESIGNATED/ELECTED OFFICE (DO/EO/US)
CONCERNING A FILING UNDER 35 U.S.C. 371

4141-2-PUS

U.S. APPLICATION NO. (if known, see 35 CFR 1.5)

10/030578

INTERNATIONAL APPLICATION NO.

INTERNATIONAL FILING DATE

PRIORITY DATE CLAIMED

PCT/US00/18712

07 July 2000

08 July 1999

TITLE OF INVENTION

"CALCIUM-CONTAINING STRUCTURES AND METHODS OF MAKING AND USING THE SAME"

APPLICANT(S) FOR DO/EO/US

STARLING, L. Brian and STEPHAN, James E.

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☒ This express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(I).
4. ☒ A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.
5. ☒ A copy of the International Application as filed (35 U.S.C. 371(c)(2))
 - a. ☐ is transmitted herewith (required only if not transmitted by the International Bureau).
 - b. ☐ has been transmitted by the International Bureau
 - c. ☒ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☐ A translation of the International Application into English (35 U.S.C. 371(c)(2)).
7. ☒ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3)).
 - a. ☐ are transmitted herewith (required only if not transmitted by the International Bureau).
 - b. ☐ have been transmitted by the International Bureau.
 - c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
 - d. ☒ have not been made and will not be made.
8. ☐ A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. ☐ An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).
10. ☐ A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11. To 16. below concern documents or information included:

11. ☐ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
12. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.23 and 3.31 is included.
13. ☐ A **FIRST** preliminary amendment.
14. ☐ A **SECOND** or **SUBSEQUENT** preliminary amendment.
15. ☐ A substitute specification.
16. ☐ A change of power of attorney and/or address letter.
17. ☐ Other items or information:

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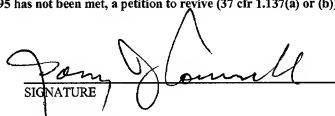
U.S. APPLICATION NO. (if known, see 37 CFR 1.5)		INTERNATIONAL APPLICATION NO. PCT/US00/18712		ATTORNEY DOCKET NUMBER 4141-2-PUS	
17. [X] The following fees are submitted:				CALCULATIONS PTO USE ONLY	
BASIC NATIONAL FEE (37 CFR 1.492(a)(1)-(5)):					
Search Report has been prepared by the EPO or JPO				\$860.00	
International preliminary examination fee paid to USPTO (37 CFR 1.482)				\$690.00	
No international preliminary examination fee paid to USPTO (37 CFR 1.482) but international search fee paid to USPTO (37 CFR 1.445(a)(2))				\$710.00	
Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid USPTO				\$1,000.00	
International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(2)-(4)				\$100.00	
ENTER APPROPRIATE BASIC FEE AMOUNT =				\$	100.00
Surcharge of \$130.00 for furnishing the oath or declaration later than months from the earliest claimed priority date (37 CFR 1.492(e)). [] 20 [] 30				\$	
CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE		
Total Claims	54 - 20 =	34	x \$18.00	\$	612.00
Independent Claims	3 - 3 =	0	x \$80.00	\$	0.00
MULTIPLE DEPENDENT CLAIMS(S) (if applicable)			+ \$270.00	\$	270.00
TOTAL OF ABOVE CALCULATIONS =				\$	982.00
[X] Applicant claims small entity status under 37 CFR 1.27.				\$	(491.00)
SUBTOTAL =				\$	491.00
Processing fee of \$130.00 for furnishing the English translation later than months from the earliest claimed priority date (37 CFR 1.492(f)). [] 20 [] 30				\$	
TOTAL NATIONAL FEE =				\$	491.00
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31) \$40.00 per property				\$	
TOTAL FEES ENCLOSED =				\$	491.00
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				charged	\$

- a. [X] A check in the amount of \$ 491.00 to cover the above fees is enclosed.
- b. [] Please charge my Deposit Account No. _____ in the amount of \$ _____ to cover the above fees. A duplicate copy of this sheet is enclosed.
- c. [X] The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 19-1970. A duplicate copy of this sheet is enclosed.

NOTE: Where an appropriate time limit under 37 cfr 1.494 or 1.495 has not been met, a petition to revive (37 cfr 1.137(a) or (b)) must be filed and granted to restore the application pending status.

SEND ALL CORRESPONDENCE TO:

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Gary J. Connell
Registration No. 32,020

Rec'd PCT/PTO 29 APR 2002 #4
10/030578

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In Re the Application of:)

STARLING, L. Brian and STEPHAN, James E.)

Int'l. Serial No.: PCT/US00/18712)

PRELIMINARY AMENDMENT

Int'l. Filing Date: 07 July 2000)

Priority Date: 08 July 1999)

EXPRESS MAIL LABEL NUMBER: EV068097444US
DATE OF DEPOSIT: April 29, 2002

For: "CALCIUM-CONTAINING
STRUCTURES AND METHODS OF
MAKING AND USING THE SAME")

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Atty. File No.: 4141-2-PUS)

TYPED OR PRINTED NAME: CONSTANCE ROBBETT
SIGNATURE: Constance Robbette

Box PCT
Assistant Commissioner for Patents
Washington, D.C. 20231

Dear Sir:

Prior to the initial review of the above-identified patent application by the Examiner, please enter the following Preliminary Amendment. No fees are believed to be due in connection with the filing of this Preliminary Amendment. In the event that any fees are due, please debit Deposit Account 19-1970.

Please amend the above-identified patent application as follows:

IN THE SPECIFICATION:

On page 1, following the title of the application and before the section entitled "Field of the Invention" please insert the following new section:

CROSS-REFERENCE TO RELATED APPLICATIONS

The present application is a national phase filing under 35 U.S.C. §371 of PCT Application Serial No. PCT/US00/18712, which claims priority under 35 U.S.C. § 119(e) from U.S. Provisional Patent Application Serial No. 60/143,333, filed July 8, 1999.

200210 025500 1000578 012902

REMARKS/ARGUMENTS

The above amendments are being submitted in connection with the national stage filing of the present Application.

Attached hereto is a marked up version of the changes made to the specification and claims by the current amendment. The attached page is captioned "Version With Markings to Show Changes Made."

Respectfully submitted,

SHERIDAN ROSS P.C.

By: 

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Date: April 29, 2002

VERSION WITH MARKINGS TO SHOW CHANGES MADE

In the Specification:

On page 1, following the title of the application and before the section entitled "Field of the Invention" the following new section has been inserted:

--CROSS-REFERENCE TO RELATED APPLICATIONS

The present application is a national phase filing under 35 U.S.C. §371 of PCT Application Serial No. PCT/US00/18712, which claims priority under 35 U.S.C. § 119(e) from U.S. Provisional Patent Application Serial No. 60/143,333, filed July 8, 1999.--

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CALCIUM-CONTAINING STRUCTURES
AND METHODS OF MAKING AND USING THE SAME

5 Field of the Invention

The invention generally relates to calcium-containing structures for use as implantable substrates, cell culture substrates, carriers of nitric oxide and a variety of other uses.

10 Background of the Invention

15 Revolutionary advances in biotechnology and genetic engineering have created enormous potential for marketing cellular by-products, including for example, proteins, including protein pharmaceuticals such as interferon, monoclonal antibodies, TPA (Tissue Plasminogen Activator), growth factors, insulin, and cells for transplantation. The demand for these products has grown tremendously and will continue to do so, creating a need for efficient methods of producing industrial quantities of cell-derived pharmaceuticals and other products. Further, the demand for efficient methods of analyzing and isolating biological products through chromatographic technology, and the need to improve bio-implantables continues to grow.

20 Research and study of cell structure and morphology are fundamental to continued progress in the diagnosis and treatment of human diseases. Numerous cell products are of vital importance therapeutically and commercially, including, for example, hormones, enzymes, viral products, vaccines, and nucleic acids. The production of these products requires large scale cell culture systems for their production.

25 Mammalian cells can be grown and maintained in vitro, but are generally anchorage-dependent, i.e., they require a solid surface or substrate for growth. The solid substrate is covered by or immersed in a nutrient medium particular to the cell type to be cultured. The nutrient medium and solid substrates are contained in a vessel and provided with an adequate supply of oxygen and carbon dioxide to support cell growth and maintenance. Cell cultures may be batch systems, in which nutrients are not replenished during cultivation although oxygen is added as required; fed batch systems, in which nutrient and oxygen are monitored and replenished as necessary; and perfusion systems, in which nutrient and waste products are monitored and controlled (Lubiniecki, Large Scale Mammalian Cell Culture Technology, Marcel Dekker, Inc., New York, 35 1990).

The primary commercial systems used for mammalian cell culture use solid matrix perfusion and microcarrier bead systems (Lubineicke, supra). The solid matrix perfusion systems utilize glass columns packed with glass beads or helices, which form a matrix as the solid substrate for cell growth. Once cells have attached to the matrix, medium is continuously recycled from a storage vessel for support of cell growth and maintenance. A similar perfusion system uses hollow fibers as the solid matrix instead of beads.

In microcarrier systems, small spheres are fabricated, for example, from an ion exchange gel, dextran, polystyrene, polyacrylamide, or collagen-based material. These materials have been selected for compatibility with cells, durability to agitation and specific gravities that will maintain suspension of the microcarriers in growth mediums. Microcarriers are generally kept in suspension in a growth medium by gently stirring them in a vessel. Microcarrier systems are currently regarded as the most suitable systems for large-scale cell culture because they have the highest surface to volume ratio and enable better monitoring and control. Nevertheless, current microcarrier culture systems have a number of serious disadvantages: small microcarrier cultures cannot be used to inoculate larger microcarrier cultures; therefore, a production facility must use other culture systems for this purpose; the cost of microcarriers is high, which can necessitate reprocessing of the microcarriers for reuse with the attendant costs; and the oxygen transfer characteristics of existing microcarrier systems are rather poor.

Specific forms of calcium phosphate ceramic have been identified for use in microcarriers to support anchorage-dependent cells in suspension. These specialized ceramics provide a material, which is biomimetic, i.e., it is composed of mineral species found in mammalian tissues, and which can be further applied to a variety of *in vitro* biological applications of commercial interest. A number of common cell lines used in industrial applications require attachment in order to propagate and need substrate materials such as microcarriers for large scale cultivation. U.S. Patent No. 4,757,017 (Herman Cheung) describes the use of solid substrates of mitogenic calcium compounds, such as hydroxylapatite (HA) and tricalcium phosphate (TCP) for use in *in vitro* cell culture systems for anchorage-dependent mammalian cells. The unique features of this technology include the growth of cells in layers many cells thick, growth of cells that maintain their phenotype and the ability to culture cells for extended periods of time.

Cheung demonstrated the application of this technology for culturing red blood cells. A current limitation of this technology is that the microcarriers are only available in a non-suspendable granular form. The density of these microcarriers further limits the ability to scale-up this technology for large bioreactors, which require a suspendable microbead carrier. Cheung, also describes the use of large substrates in monolithic forms for the culture of cells, but he does not identify methods for producing large area monoliths conforming to the contours and sizes of tissues to be replaced *in vivo* or grown *in vitro*. A complementary system using an aragonite (CaCO₃) is described in U.S. Patent No. 5,480,827 (G. Guillemin et al). Although this patent also mentions the importance of calcium in a support system for mammalian cell culture, the calcium compound was not in a suspendable form. Likewise, Guillemin et al do not identify methods for producing large area monoliths conforming to the contours and sizes of tissues to be replaced *in vivo* or grown *in vitro*.

The concept of fabricating a suspendable microcarrier bead with a minor component of glass was discussed by A. Lubiniecki in Large-Scale Mammalian Cell Culture Technology in which a minimal glass coating was applied to a polymer bead substrate by a chemical vapor deposition process or low temperature process. This approach also was disclosed in US Patent No. 4,448,884 by T. Henderson (see also US Patent Nos. 4,564,532 and 4,661,407). However, this approach primarily used the polymer bead substrate to maintain suspendability.

An example of the use of non-suspendable or porous ceramic particles for cell culture is taught by U. S. Patent No. 5,262,320 (G. Stephanopoulos) which describes a packed bed of ceramic particles around and through which oxygen and growth media are circulated to encourage growth of cells. U. S. Patent No. 4, 987,068 (W. Trosch et al.) also teaches the use of porous inorganic (glass) spheres in fixed bed or fluidized bed bioreactors. The pores of the particles act as sites for the culture of cells. Conversely, Richard Peindhl, in U.S. Patent No. 5,538,887, describes a smooth surface cell culture apparatus which would limit cell attachment to chemical adhesion and prevent mechanical interlocking.

Macroporous glass beads also have been reported by D. Looby and J. Griffiths, "Immobilization of Cells In Porous Carrier Culture", Trends in Biotechnology, 8:204209, 1990, and magnesium aluminate porous systems by Park and Stephanopolous, "Packed

Bed Reactor With Porous Ceramic Beads for Animal Cell Culture", Biotechnology Bioengineering, 41: 25-34, 1993. Fused alumina foams have been reported by Lee et al, "High Intensity Growth of Adherent Cells On a Porous Ceramic Matrix." Production of biologicals from Animal Cells in Culture, editors, R.E. Butterworth-Heinemann et al., pp. 400-405, 1991, and polyurethane foam by Matsushita et al., "High Density Culture of Anchorage Dependent Animal Cells by Polyurethane Foam Packed Bed Culture Systems", Applied Microbiology Biotechnology, 35:159-64, 1991.

Fluidized bed reactors have been used for cell culture as reported by J.M. Davis (editor), Basic Cell Culture, (Cartwright and Shah), Oxford University Press, New York, 1994, but require carrier systems with densities between 1.3 and 1.6 g/cc. According to Cartwright (J.M. Davis, supra.), generally, in fluidized beds, cells do not grow on the exterior surface of carriers where they would be dislodged by interparticle abrasion. Instead, as with macroporous microcarriers, they colonize the interior pores where they proliferate in a protected microenvironment. As examples, (Cartwright, supra, p. 78) cell carriers used in fluidized beds include glass beads (Siran by Schott Glass), and collagen microspheres produced by Verax. Cartwright also describes other conventional microcarriers weighted with TiO₂ (Percell Biolytica products) and LAM-carrier polyethylene beads weighted with silica.

Hydroxylapatite and calcium phosphates have been used for implant applications with and without bone mixtures and bone growth factors. For example, Jarcho, Dent. Clin. North Am. 30:25-47 (1986) describes implanting of calcium phosphates to augment bone, while Ripamonti et al, MRS Bulletin 36-39 (November, 1996) describes augmentation of bone with bone morphogenic proteins (BMP's), including TGF-beta, BMP's 108, OP-1 and 2, and demineralized bone matrix with and without hydroxylapatite. Other growth factors applicable for bone augmentation are also set forth by Lane et al, Clinical Orthopaedics and Related Research, 367S, S107-117 (October 1999), and include recombinant human bone morphogenetic protein (rhBMP), fibroblast growth factor (FGF), acidic fibroblast growth factor (aFGF), basic fibroblast growth factor (bFGF), insulinlike growth factor (IGF), platelet derived growth factor (PDGF), and growth differentiation growth factor (GDF). Various types of calcium implants, including tricalcium phosphate, hydroxylapatite, calcium phosphate and calcium carbonate, are listed in Han et al., J. Western Soc. Periodontology:Perio

Abstracts 32(3):88-108 (1984). The use of dense and porous hydroxylapatite with and without grafted bone is reported in Matukas et al., J. Neurosurgery, 69:514-517 (1988). Similarly, Small et al., Int'l J. of Oral Maxillofacial Implants, 8(5):523-528 (1993) describes hydroxylapatite particles used with freeze dried bone to augment bone for implants. Also, Hollinger, United States Army Institute of Dental Research Information Bulletin, 4(2) (Winter, 1990), lists grafting materials that include various commercial types of hydroxylapatite, tricalcium phosphates and bone grafts. Finally, several investigators have demonstrated the culturing of marrow tissues, stem cells, and periosteal-derived cells on calcium phosphate materials, which have subsequently been implanted in animal models to produce the formation of bone and/or cartilage tissue by osteochondrogenic induction. These investigators include Nakahara et al, Clinical Orthopaedics, (276):291-8 (Mar, 1992); Grundel et al, Clinical Orthopaedics, (266):244-58 (May 1991); Toquet et al, Journal of Biomedical Research, 44(1):98-108 (Jan 1999); Bruder et al, Journal of Bone and Joint Surgery, 80(7):985-96 (July 1998). However, none of these publications describe the use of hollow calcium-containing microspheres as implantable substrates. All of these approaches to the culture of anchorage dependent cells suffer from either the inability to grow cells in a suspendable environment or to allow an encapsulated volume in which cells can be grown or have a proven viability as an implant material. Furthermore, the mass of material implanted as a carrier of cellular components is generally in excess of what can be resorbed in a ready manner.

Bonding agents applied as coatings can be used in conjunction with hollow microspheres are sited in WO98/43558, and include both polymeric and calcium based cements. Resorbable polymers which are applicable for orthopedic augmentation applications have been summarized by Behravesh et al, Clinical Orthopaedics and Related Research, 367S, S118-125 (October 1999), and include polylactic acid, polygalactic acid, polycaprolactone, poly α -hydroxy esters, polyphosphazenes, polyanhydrides, and polypropylene fumarate. US Patent No. 5,522,893 (Chow et al), sets forth a review of methods for making calcium phosphate cements, which can be employed for bone augmentation applications. Likewise, US Patent Nos. 4,612,053 (Brown et al) and 5,047,031 (Constantz et al) establish alternative and complementary methods of calcium phosphate cement formulation. Wright Medical Technology, Inc.

(Memphis, Tennessee) has also produced a granular calcium phosphate for bone augmentation called Osteoset® and calcium sulfate used as a cement to incorporate a demineralized bone matrix called Allomatrix™ and further cites in its literature that calcium sulfate has been used for over 100 years as a bone void filler. Generally, cements used for bone augmentation applications lack sufficient porosity to allow for optimal bone in-growth and release of biological agents or live cells.

An excellent review of nitric oxide in biological applications is cited in the Encyclopedia of Inorganic Chemistry, Volume 5, 2482-2498, John Wiley & Sons (1994) and Nitric Oxide in Health and Disease, by J. Lincoln, Cambridge University Press, Cambridge, New York & Melbourne, 1997. These references cite that nitric oxide (NO) is naturally produced in the body and is used on the cellular level as a defense against invading organisms, as a regulator of vascular tone and as a neuronal signaling agent. The concentration of nitric oxide and the presence of biological inhibiting agents or promoting agents determine the ultimate outcome, whether promoting tissue proliferation, health or tissue destruction.

According to these references, there are three types of cellular precursors to NO. They are all identified as nitric oxide synthases (NOS): Type I is associated with neurones, Type II is associated with a variety of cell types, but is primarily associated with host response to infection or invading organisms. Type III is primarily associated with endothelial cells.

NO has been implicated in increasing blood flow by arterial dilation, which can beneficially impact heart function, penile erection, and maintenance of blood supply to peripheral organs (Ziche et al, Journal of Clinical Investigation, 94, 2036-44. According to J. Lincoln, Nitric Oxide in Health and Disease, Cambridge University Press, Cambridge, New York & Melbourne, 1997, NO and NO antagonist have been used to treat asthmatic conditions. Likewise, NO has been used to increase nerve function (Schuman et al, Annual Reviews of Neuroscience, 17, 153-83, 1994). Conversely, in larger concentrations, NO has been documented to be cytotoxic to tumor formations (Rocha et al, International Journal of Cancer, 63, 405-11, 1995). NO has also been suggested to play a number of roles in renal function including renal blood flow, renin secretion and pressure induced natriuresis and diuresis (Bachmann et al, American Journal of Kidney Diseases, 24, 112-29, 1994). NO has also been implicated as a growth

factor (J. Lincoln, Nitric Oxide in Health and Disease, Cambridge University Press, Cambridge, New York & Melbourne, 1997). Nitric oxide can also be used to treat pathogens and other invading organisms as set forth by James, S.L., Microbiological Reviews, 59, 533-47, 1995.

5 Tam et al, Life Sciences, 51, 1277-84, 1992 describes a method of producing NO from sodium nitrite and hydrochloric acid. NO can also be purchased as a purified gas from commercial suppliers such as Matheson Gas Products, Cucamonga, CA. Also, Manahan in Environmental Chemistry, 6th Edition, Lewis Publishers (1994) describes methods for the formation of NO at high temperatures from N₂ and O₂ in controlled
10 atmospheres. Ishii et al, American Journal of Physiology, 261, 598-603, 1991 describes a method of producing NO from sodium nitrite and hydrochloric acid.

The treatment of biological disorders with polymeric compounds binding NO compositions is set forth in U.S. Patent No. 5,718,892 (Keefer et al). This patent further demonstrates the usefulness of delivering NO for other medical applications. Other precursor forms of NO compounds include nitric oxide synthase or L-arginine. Also, superoxide dismutase causes an increase in the production of free NO as cited by Hobbs et al, Proceedings of the National Academy of Sciences USA, Nov. 8: 91, (23): 10992-6, 1994. Furthermore, NO is a physiological messenger and cytotoxic agent dependent on other mediating agents (J. Lincoln, Nitric Oxide in Health and Disease, Cambridge University Press, Cambridge, New York & Melbourne, 1997).

NOS (nitric oxide synthases) Types I and III depend on elevated Ca²⁺ levels to become activated, and Type II is regulated to a lesser degree by the presence of Ca²⁺ (Jordan et al, Surgery, 118, 138-45, 1995). These references together indicate that all three types of nitric oxide synthases are regulated to some degree, either directly or
25 indirectly, by the presence of calcium. Also, Schuman et al, Annual Reviews of Neuroscience, 17, 153-83, 1994 showed that phosphorylation of Type I NOS may form an additional mechanism for regulating its activity. Methods for extraction and purification of the three types of NOS are given by Fosterman et al, Methods in Enzymology, 233:258-64, 1994. It should also be noted that NOS is regulated by the
30 presence of NO according to Rengasamy et al, Molecular Pharmacology, 1993, Jul. 44 (1): 124-8.

5 The current technology for the delivery of nitric oxide for medical applications generally relies on polymers for implant applications and does not anticipate the need for the presence of calcium and/or phosphate to regulate nitric oxide or nitric oxide precursors. Likewise, for non-implant applications nitric oxide components are generally delivered by inhalation, or as free pharmaceutical agents. Also, nitric oxide or substances containing nitric oxides, have not been previously encapsulated in or used in conjunction with ceramic or glass materials for the delivery of nitric oxide for medical applications.

10 Accordingly, a need exists for new implantable substrates as well as methods of effectively delivering NO to a desired site. The present invention satisfies this need and provides related advantages as well.

Summary of The Invention

15 In one aspect of the invention, the disadvantages of the prior art are overcome by the present invention through the use of hollow calcium containing structures mixed with bone or bone derived substances. Thus, the hollow calcium containing structures function to supplement bone or bone derived substances and are, therefore, referred to as bone graft extenders.

20 Likewise, the same hollow calcium containing structures, with or without bone or bone derived materials, can be used in conjunction with bonding agents as materials for joining hollow microspheres or other hollow forms in a monolithic structure. Likewise, the bonding materials used to bond the hollow forms can also be used to bond to tissues at the implant site. These bonding agents can be either polymeric materials or calcium containing cements. Useful polymeric materials include, for example, polylactic acid, polyglycolic acid, polycaprolactone, poly α -hydroxy esters, polyphosphazenes, polyanhydrides, and polypropylene fumarate. The calcium in the cements is preferably calcium phosphate, calcium sulfate or a mixture thereof. The desired amount of calcium containing cement ranges from about 5% to about 75%, and more preferably from about 10% to about 50% by volume of the total composition.

30 Furthermore, this invention sets forth a method for encapsulating nitric oxide gas or nitric oxide containing compounds, or precursors to nitric oxide (also referred to herein as "nitric oxide forming compounds") in conjunction with calcium containing

materials for medical therapeutic applications. This invention also provides methods for coating surfaces of calcium containing compounds or mixing calcium containing compounds with precursors of nitric oxide, and/or antagonists or activators to nitric oxide formation.

Also described in this invention is a method for fabricating calcium containing substrates with surfaces, which conform to tissue surfaces in the body requiring replacement or augmentation. These substrates are fabricated from the cast tape process to produce sheet structures, which are flexible in the unfired state, and can be contoured by firing on refractory shapes, which conform to the site of implantation. Furthermore, the invention comprises a method for using the cast tape components and building them up in layers to produce the desired shape as substrates for cell culturing applications. The cast tape components are built up in the same manner as three dimensional topographic structures. In each case these substrates can be implanted with or without the cultured tissue to augment the desired implant site.

Detailed Description of the Invention

The invention relates to the use of hollow calcium-containing microspheres for a variety of purposes. Such uses include bone graft extenders, bonding agents, cell culture substrates and carriers of nitric oxide.

A. Bone Graft Extender

In one aspect, the invention relates to methods of making a bone grafting mixture in which the calcium-containing microspheres or other hollow microstructures are used to extend bone material. The methods are generally accomplished by:

- (a) obtaining hollow calcium-containing microstructures; and
- (b) mixing the hollow calcium-containing microstructures with a bone material to form a bone grafting mixture.

Useful hollow calcium-containing microstructures for these methods include the hollow calcium phosphate (CaP) microspheres as described in PCT publication WO 98/43558, incorporated herein by reference. Particularly useful calcium phosphate for making the hollow CaP microstructures includes hydroxylapatite, tricalcium phosphate (alpha- and/or beta-), dicalcium phosphate (hydrous and/or anhydrous forms), and

tetracalcium phosphate. These hollow microstructures can be fabricated in porous or dense form as described in PCT publication WO 98/43558.

Alternatively, hollow calcium oxide/calcium hydroxide microstructures or mixtures of hollow CaP and calcium oxide/calcium hydroxide microstructures can be used. These can be prepared in the same manner as described for calcium phosphate microspheres in PCT publication WO 98/43558 or as described in U.S. Patent No. 4,133,854, incorporated herein by reference, which describes methods for making small hollow glass, metal or plastic microspheres.

The hollow calcium-containing microstructures are mixed with a mixture containing bone (referred to herein as "bone mixture" or "bone material"). The bone mixture can be comprised of cancellous bone and/or de-mineralized bone matrix (DBM), which is made from tissue bank processed freeze drying methods as described, for example, in Friedlaender et al., Osteochondral Allografts, pp 181-192 (Little, Brown & Co., Boston/Toronto 1983). The bone mixture and hollow microspheres are either dry mixed in a container with a spatula or other mixing tool or device. The components can be mixed in saline, distilled water, blood, other physiologically-acceptable fluid or a mixture thereof. In addition, bone growth factors can be mixed with this mixture. Useful bone growth factors are listed in Repamonti et al., MRS Bulletin, 36-39 (November, 1996).

The volume amount of bone material in the bone grafting mixture can range from about 5% to about 95% or more typically from about 50% to 75%. The amount of bone mixture depends on the extent of ingrowth of bone desired within the hollow microspheres and, if desired, the subsequent resorption of the hollow microspheres. Both the extent of bone in-growth and resorption of the microspheres can be controlled by adjusting the calcium content and porosity of the hollow microspheres, which can be readily determined by those skilled in the art and from the teachings of PCT publication WO 98/43558.

The present invention further provides methods for using the bone grafting mixture in which the mixture is implanted in bone defects or in surgical sites where bone growth or augmentation is desired. Those skilled in the art can readily determine the amount of bone grafting mixture to be implanted depending on several factors including, for example, the extent and location of bone defect or surgical site to be augmented. The

advantages of the present methods over known mixtures of bone material and non-hollow particles is that bone growth can also occur within the hollow microstructures rather than around the non-hollow particles, thus providing a better matrix for bone growth. Also, the hollow structure of the calcium material provides an optimal structure for maximizing bone in-growth and eventual replacement of the calcium containing material, while maintaining mechanical integrity of the implanted mixture. In any event, the hollow structure provides a lower mass of calcium containing material to be replaced at the implant site by host tissues. In addition, the hollow microspheres can undergo subsequent resorption by adjusting the calcium content and porosity of the microspheres depending on the desired rate of resorption.

B. Bonding Agent

Another aspect of the invention relates to calcium-containing bonding agents that can be used to produce the calcium-containing microstructures or for inclusion in the bone grafting mixture described above. Particularly useful bonding agents include various calcium cements containing, for example, calcium phosphate, calcium sulfate, calcium oxide/calcium hydroxide or a mixture of CaP and calcium oxide/calcium hydroxide.

Typically, the cement is comprised of an anhydrous or semi-anhydrous form of the calcium component, for example, an anhydrous or semi-anhydrous form of calcium phosphate or calcium sulfate. Preferably, the cement component of the cement/bone grafting mixture ranges from about 5 to about 75 volume percent, and more preferably from about 10 to 50 percent by volume, for the total mixture. One skilled in the art can readily determine the desired percentage depending on, for example, the intended use and if resorption is desired.

An example of a calcium containing cement is described in U.S. Patent No. 5,522,893 (Chow), incorporated herein by reference. The calcium phosphate cement is prepared by mixing tetracalcium phosphate and dicalcium phosphate anhydrous at a molar ratio of 1:1, and mixing with 25 mmol/L phosphoric acid at a powder to liquid ratio to 4.0 at ambient temperature. This mixture is subsequently stirred into the hollow microspheres at 50 percent by volume. The mixture is delivered to the implant site and allowed to set by hydrothermal curing. A similar example of bonding the hollow

microspheres would be the replacement of the calcium phosphate cement with a mixture of plaster-of-Paris (hemi-hydrate salt of calcium sulfate) in an aqueous or acidic media, such as distilled water for example.

Also, the bonding agent can be a mixture of polymeric material and hollow calcium containing microstructures. Useful polymeric materials include polylactic acid, polyglycolic acid, polycaprolactone, poly α -hydroxy esters, polyphosphazenes, polyanhydrides, and polypropylene fumarate. A specific example of a bonding agent is based on mixing crosslinkable polyanhydride polymers by photo polymerization as described by Muggli, D.S. et al., Macromolecules, 31,4120-25, (1998). In this example, 10 percent by volume of said polyanhydride polymer is mixed with 90 percent by volume of hollow microspheres. The mixture is delivered to the implant site and cured *in situ* using a photo polymerizable light source.

Hollow calcium phosphate microspheres can also be fabricated from calcium cements, which have the advantage of being able to bond to each other by the addition of aqueous media. For example, these can be prepared in the same manner as described for calcium phosphate microspheres in PCT publication WO 98/43558 with the exception of using tetracalcium phosphate and dicalcium phosphate anhydrous materials as starting materials in a molar ratio of 1:1 in place of hydroxylapatite or tricalcium phosphate. (See US Patent No. 5,522,893 (Chow)). These materials must be applied to wax or other organic microspheres in powder form or other non-aqueous form (solvent based, such as methyl ethyl ketone or terpene, for example) form to prevent hydration of the starting powders. The resulting coated spheres must be heated to decompose the organic components, and, in the case of a solvent processed material, to remove the solvent. The resulting spheres can be bonded by mixing with 25mmol/L phosphoric acid in a sphere to liquid ratio of 4.0 at ambient temperature.

The benefit of adding a bonding agent to the above mixtures is that they gain mechanical integrity and can better conform to the site of implantation. Likewise, they can bond with the tissue at the implant site. Also, by adjusting the amount of bonding agent, a high degree of open porosity can be maintained, which enhances bone in-growth.

C. Nitric Oxide Carriers

A further aspect of the present invention relates to the beneficial effects of nitric oxide for enhancing tissue growth and proliferation. More particularly, the invention relates to the (1) containment of NO gas or (2) liquid containing NO in concentrations of the desired amount or (3) precursor compounds to NO all of which are intended to provide a therapeutic effect or enhance proliferation of cells or tissues during cell culturing through action as a growth factor. Such concentration can be controlled by dilution of the entrained NO with inert gases such as argon. Likewise the concentration of NO in the liquid can be controlled by similar methods.

The primary reason for using a calcium substrate is that NOS Types I & II depend on elevated Ca^{2+} levels to become activated. Likewise, Type III also is regulated to a lesser degree by the presence of Ca^{2+} . For this reason, implantable substrates, including for example calcium phosphates, calcium oxide, and Bioglass[™] (calcium phosphate glasses) are useful candidates for the delivery of NOS to the site of implantation or during cell culturing. Likewise, these calcium-containing substrates are useful candidates for the delivery of cell types that produce the required NOS type at the implant site. Furthermore, agents such as L-arginine and aminoguanidine can also be delivered as agents which mediate the formation of NO within the cells. However, the mechanism for producing the desired response is also dependent on the resulting concentration of NO and the presence or absence of other known activators and inhibitors. These precursors, inhibitors and activators can be delivered simultaneously or in subsequent deliveries to the implant site or cell culture media.

Accordingly, the present invention relates to novel methods for delivering nitric oxide to localized sites, for example, implant sites or bioreactors for the case of cell culture. The methods are generally accomplished by initially encapsulating nitric oxide or precursor to NO within or on a calcium phosphate or other biocompatible material, including, for example, the porous or hollow microstructures described above or calcium-containing particulates.

For example, NO gas or liquids containing NO can be encapsulated in hollow portions of calcium/phosphate glass and placed or injected into the implant site, and the encapsulated nitric oxide is subsequently released *in vivo* or *in vitro* by resorbing the implantable material for therapeutic purpose. Likewise, the NO gas or gas containing

liquid could be infiltrated into porous or hollow calcium/calcium phosphate particles which are subsequently sealed with a bioresorbable polymer such as polylactic acid, polyglycolic acid, polycaprolactone, poly α -hydroxy esters, polyphosphazenes, polyanhydrides, and polypropylene fumarate or other biocompatible, bioresorbable and/or biopermeable polymer. Subsequent to implantation the polymer would either resorb or allow for the release of the NO. The gas can be incorporated in the glass fiber, bead or other hollow form by having a saturated atmosphere of the gas, in which the a molten form is enclosed around the gas, or the hollow form openings are pinched shut by melting the glass. In a manner similar to ampoules, which contain liquid solutions of ammonia in small, sealed glass containers, liquids containing NO can be prepared for implant site delivery. Liquids such as water containing NO could be used to saturate porous or hollow structures which are subsequently sealed in place by organic or polymer coatings as previously cited, or by entrapment by localized melting of hollow structures openings such that they are closed off by the hosting material.

Biological glasses (e.g., Bioglass[®]) can be used for implant applications. Biological glasses are sodium silicate based but incorporate CaO and P₂O₅ in their composition. Typical compositions are described in U.S. Patent Nos. 5,981,412 (Hench et al) and 4,608,350 (Howard), incorporated herein by reference. The properties of molten glass are conducive to hollow shape fabrication techniques (such as light bulbs) known to those skilled in the art are described in The Technology of Glass and Ceramic: An Introduction, Glass Science Technology, No. 4, J. Hlavac, Elsevier Scientific Publishing Co., New York & Amsterdam (1983), incorporated herein by reference. Methods for making glass microspheres are detailed in U.S. Patent Nos. 5,176,732 (Block et al), 4,133,854 (Hendricks), and 4,767,726 (Marshall), all incorporated herein by reference. Furthermore, methods for introducing a gas into glass microspheres are given in U.S. Patent Nos. 4,257,798 (Hendricks et al) and 4,547,233 (Delzant), both incorporated herein by reference.

Other methods for making gas-containing structures for gases other than NO are readily known to those skilled in the art, including, for example, the methods described in U.S. Patent No. 4,257,798, incorporated herein by reference, which describes methods for introducing gases into microspheres. Alternatively, the calcium and/or calcium phosphate material could also be encapsulated in a particulate form in a carrier polymer,

thereby gaining the activating nature of the Ca^{2+} released from the calcium containing compound.

The delivery of NO or other NO inducing agents or cells also has application in the in vitro culture of cells (cell culture/bioreactor applications) and tissues and bio-products derived from them. Examples of calcium containing structures with some form of NO are provided in the Examples below.

All of the compositions described in the examples can be used as medical therapeutic agents or enhancements for cell culture applications.

D. Substrate for Cultured Cells

The present invention further relates to methods of culturing cells on calcium-containing substrates, conforming to tissue to be replaced *in vivo*. A variety of calcium compounds can be used as cell culture substrates, including, for example, calcium oxide, Bioglass, dicalcium phosphate, tricalcium phosphate, hydroxylapatite, tetracalcium phosphate, and the like.

The substrates can be in any desired geometrical form or shape, including, for example, microspheres, planar, tubular, three dimensional scaffold, or other geometrical form that complements the missing tissue at the site to be constructed. The microspheres can be produced, for example, as described in WO 98/43558, while the other geometrical shapes can be fabricated by any method known to those skilled in the art, including customary ceramic powder fabrication methods, such as, for example, tape casting, slip casting, gel casting, extrusion, isostatic pressing, tape lamination and molding processes such as injection or transfer molding. After the forming step, the substrate in the desired geometric shape is sintered to the desired porosity, density and strength state. For example, the substrates can be produced by ceramic processing methods in which various densities can be obtained through firing as described in Reed, Principles of Ceramic Processing, 2d ed. (John Wiley & Sons, NY, 1995).

Methods for fabricating cast tape ceramic components from oxide materials are well known to those skilled in the art of ceramic component fabrication, and is described for example in Reed, Principles of Ceramic Processing, second edition, pp 525-540 (John Wiley & Sons, NY, 1995), incorporated herein by reference. For example, thin, flexible green sheets of CaP compositions can be produced with either a batch or continuous cast

tape process. Relevant CaP compositions are comprised of hydroxylapatite, tribasic calcium phosphate, dicalcium phosphate (hydrous or anhydrous), calcium carbonate, calcium oxide, Bioglass or any mixture thereof. The initial step for cast tape processing requires a concentrated, milled ceramic slurry containing dispersed CaP powders mixed with a relatively high concentration of polymeric binders and plasticizers in a solvent medium system. A thin, flexible CaP tape is formed when the ceramic slurry flows beneath a blade (known as a doctor blade in the industry) forming a film on a moving carrier of a high surface quality polymeric carrier such as a polyester film (Mylar[®]), which is then dried in a controlled temperature environment.

In a batch process, thin sheets of CaP ceramic can be formed by pouring the ceramic slurry into a doctor blade mounted in a box assembly and moving the blade assembly over a stationery carrier film such as Mylar[®] supported by a flat ground surface plate made of glass or metal. In the batch process, the cast tape is then allowed to dry to remove the solvent medium. The resulting dried cast tape is flexible with a smooth surface texture, which allows for green ceramic sheets to be separated from the polymeric carrier film, which are then cut to size. In a continuous process, the doctor blade box assembly is held in a fixed position and the carrier film is continuously run underneath the doctor blade for depositing a thin film of ceramic slurry. The ceramic slurry is then dried in a controlled heated atmosphere prior to stripping of the ceramic cast tape. For both processes, the green-state CaP tape can be handled as separate sheets or can be rolled for storage prior to cutting into sized shapes.

With the high concentration of polymeric binders and plasticizers in the green-state ceramic tape, individual sized shapes may be sintered to form flat, thin substrates. Also, individual layers of tape can be formed over contoured surfaces by subsequently heating the cast tape to thermoplastically deform the cast tape followed by cooling to establish the contoured shape. This process is possible as a result of the flexible, rubbery nature cast CaP tape layers that adapt to the shape of specific forms to be replicated. After the contouring process to establish shape, a low temperature treatment of approximately 300°C is used to partially decompose the binder and to rigidize the CaP sheet structure. If needed, this process may benefit from a refractory support structure during thermal curing of the ceramic tape, which can be fabricated from plaster-of-Paris or a commercial castable cement. A castable refractory material may also be employed

to support the contoured CaP cast tape shape during the high temperature sintering process, which occurs over the temperature range from approximately 1100°C to 1400°C.

Also, individual layers of green-state CaP cast tape may be laminated to bond individual sheets in forming a monolithic structure which is then sintered. Three dimensional structures can then be fabricated starting with individual sheets of cast tape. Using a computer generated model, three dimensional structures can be sectioned into separate thin layers by computer simulation. These dissected thin layers are then fabricated from cast tape sheets. The exact outline configuration of each cast tape sheet is defined by a laser generated contour using computer-aided design methods established in the industry. Each cast tape outline is created using automated control of a laser beam to precisely cut each layer of tape representing a dissected section of the actual model to be recreated in an appropriate calcium containing composition. The individual layers of tape are then assembled and laminated with aid of heat and pressure (with or without solvent) to form an actual replica of a three-dimensional contoured shape. By characterizing the shrinkage of the cast tape during sintering, the size of each cast tape shape in the assembled unit is adjusted to compensate for shrinkage.

The primary steps that comprise the basic cast tape process for preparing thin sheets to be used as substrates for flat geometries, contoured surfaces or laminated three-dimensional structures are comprised of the following:

- Ball milling of CaP powder, binder(s), plasticizer(s), deflocculant (dispersant) and solvent(s).

- Mixing step

- De-airing

- Filtering

- Tape casting

- Drying

- Separation of cast tape from carrier film

- Cutting shapes from cast tape sheets to size

The specific CaP composition can be tailored for exacting properties defined by chemistry and processing conditions, which as an example, defines its ability to resorb in cell culturing either *in vitro* or *in vivo* for implant applications.

The cast tape process can be successfully accomplished using several types of binder systems, depending on whether an aqueous or non-aqueous solvent system is employed. A typical solvent system is comprised of ethyl or methyl alcohol blended with methyl ethyl ketone or toluene. For this solvent system, a common binder used in ceramic tape formulations is polyvinyl butyral (Butvar[®] PVB B72 or B90) and a phthalate plasticizer (Santicizer[®] 160). An acrylic (Acryloid[®] B-7 in methyl ethyl ketone solvent) has been shown to be an alternative to PVB for improved binder burn-out with little or no measurable ash content. As a dispersant in solvent systems, Menhaden fish oil has been shown to work well in deflocculating the CaP particles and to improve release of cast tape from the polymer film carrier.

The use of aqueous based acrylic emulsions provides an alternative and effective processing path for minimizing the health effects and hazards of organic solvents such as toluene and methyl ethyl ketone. One system that has been identified is based on Rohm & Haas' Duramax family of acrylic emulsions. The ability to obtain a high solids loading of CaP powder in water begins with the use of acrylic dispersants such as Duramax D-3005 or D-3021 or R.T. Vanderbilt's well know Darvan family of dispersants such as Darvan C or Darvan 7. One example of an acrylic cast tape system is comprised of:

100 grams of calcium phosphate processed powder (HA/TCP mixture)
55 grams of de-ionized water
0.86 grams of Duramax D-3021
Ball mill for 2-3 hours, then mix by agitation at 100-300 rpm:
12.7 grams of Duramax B-1001 ceramic binder (55% solids, 7% solids basis)
3.0 grams of Polyethylene Glycol 300 MW (Polysciences)
De-airing step followed by tape casting.

Other acrylic emulsion resins can be blended with or substituted for Duramax B-1001 to adjust the T_g (glass transition temperature); thereby, increasing or decreasing the flexibility of CaP cast tape. Such alternative resins include Duramax B-1080 to include the flexibility of CaP cast tape. Duramax B-1080 is also an effective binder for producing ultra-thin cast tapes having a thickness less than 50 micrometers. Other

acrylic resins known in the art for casting ceramic tapes include Duramax B-1080 and B-1000.

Alternatively, the substrate of the present invention can be produced by melting a glass composition containing calcium phosphate and fabricating the desired structure using well established glass forming methods based on melt processing techniques known to those skilled in the art, including for example, the methods described in The Technology of Glass and Ceramic: An Introduction, Glass Science Technology No. 4. J Hlavac. Elsevier Scientific Publishing Co. 1983. NY, Amsterdam.

Methods for using the cells and tissues cultured on the calcium-containing substrates are also within the present invention. In these methods, the desired geometric shape of the cultured cells/tissues depends on the tissue to be replaced, repaired or augmented in the patient. More specifically, the tissue that is replaced, repaired or augmented can include bone, cartilage, tendon, ligament, skin, muscle, kidney, heart, pancreas, or cardiovascular, neural, urological, respiratory, intestinal, endocrine and liver tissues. For example, tubular structures can be used for veins, arteries, or other forms to conform to the implant sites such as cartilage and ligaments.

The cells or tissues are grown on the calcium-containing substrates according to cell culture conditions known to those skilled in the art or as described in Example 6 below. Once a desired amount of cell growth has occurred, the cultured cells/tissues that have the desired geometric shape can be physically or chemically removed from the calcium-containing substrate and transplanted to the defect or surgical site to be augmented according to procedures known to those skilled in the art. Alternatively, the cultured cells/tissues can be transplanted with the calcium-containing substrate to the defect or surgical site to be augmented. Methods of transplanting are known in the art, including, for example, those methods described in Synthetic Biodegradable Polymer Scaffolds by Anthony Atala et al, Editors, (Birkhauser Publishing, Boston 1997) and Frontiers in Tissue Engineering, Charles W. Patrick Jr. et al, Editors (Elsevier Science Ltd. 1998), both incorporated herein by reference.

The following examples illustrate, but are not intended to limit, various embodiments of the present invention.

EXAMPLE 1

NO Gas Containing Structures

NO gas obtained from Matheson Specialty Gas or NO produced from methods as previously described above is introduced into an evacuated chamber and is backfilled while forming hollow glass microspheres as described in U.S. Patent No. 4,257,798 (Hendricks et al). In this example, hollow glass microspheres are fabricated in a glass composition according to U.S. Patent No. 5,981,412 (Hench et al), which is approximately 47% SiO₂, 24% CaO, 24% Na₂O and 5% P₂O₅. The glass composition is initially melted above 800°C and processed according to Hendricks to produce NO containing hollow microspheres. This method may be improved upon by making NO gas *in situ* by introducing nitrogen and oxygen gases at temperatures greater than 800°C to form NO during the glass hollow structure fabrication.

EXAMPLE 2

NO Gas Containing Structures

NO gas obtained from Matheson Specialty Gas or NO produced from methods as previously described is introduced into an evacuated glass tube or hollow fiber. After filling with NO gas, the glass tube or hollow fiber is cut with a Nichrome wire, which is resistance heated above 800°C and is rapidly passed through the glass structure to seal the NO gas within the segmented structures without decomposition of the encapsulated NO gas. This technique can also be used to encapsulate NO aqueous containing solutions by introducing the liquid into the glass tubes or hollow fibers.

EXAMPLE 3

NO Gas Containing Structures Sealed with Polymer

A porous calcium containing structure such as a mixture of 75% by weight of tricalcium phosphate and 25% by weight of hydroxylapatite is synthesized into 500 micron diameter hollow microspheres or particles. The structure is then sintered above 1000°C to produce porous structures. These microspheres or particles are subsequently placed in an evacuated chamber and backfilled with NO gas as previously described and at one atmosphere pressure in a sealed, heated vessel having a stirrer. The vessel containing the NO treated microspheres or particles is heated above the melting point of

the polymer. The melted polymer such as polycaprolactone is then titrated into the sealed vessel as the microspheres or particles are stirred. A sufficient amount of polymer is titrated onto the hollow microspheres or particulates to the extent that agglomeration of the particles is initiated. The amount of polymer can be adjusted to change the rate of biodegradation. Stirring is continued while the polymer coated agglomerates are allowed to cool to form a sealed surface on the agglomerated microspheres or particulates. Also, solution polymerization in a controlled NO atmosphere can be used instead of melting a polymer for producing a polymeric coating encapsulating NO with hollow microspheres or particulates.

EXAMPLE 4

NOS or NO Precursor Coatings on Calcium Containing Hollow Microstructures or Particulates

A porous calcium containing structure such as a mixture of 75% by weight of tricalcium phosphate and 25% by weight of hydroxylapatite is synthesized into 500 micron diameter hollow microspheres or particles. NOS is prepared by methods set forth by Fosterman et al, Methods in Enzymology, 233, 258-64, 1994. The desired NOS type, such as Type I, is mixed with the calcium containing hollow microstructures or particulates to form a coating upon same. Likewise, L-arginine can be used as an NO precursor or with superoxide dimutase as an enhancer of biological activity and mixed the calcium containing hollow microstructures or particulates to form a coating upon same as described above. A further modification to Type II NOS is the incorporation of NO inhibitors such as aminoguanidine to regulate the release of NO as specified in Nitric Oxide in Health and Disease, by J. Lincoln, Cambridge University Press, Cambridge, New York & Melbourne, p. 153, 1997.

EXAMPLE 5

Polymeric Materials, Which Release NO Used in Conjunction with Calcium Containing Compounds

A calcium containing structure such as a mixture of 75% by weight of tricalcium phosphate and 25% by weight of hydroxylapatite is synthesized into 500 micron diameter hollow microspheres or particles. The said structure is mixed with a polymeric

material, which releases NO as described by U.S. Patent No. 5,994,444 (Trescony et al). For instance, a polylactic acid and polyglycolic acid copolymer with inorganic nitrite dispersed within the copolymer is then mixed with greater than 5 percent by volume of the said calcium containing structure. Said mixture can be used with or without NOS additions.

EXAMPLE 6

Culture of Osteoblasts on Calcium Phosphate Microcarriers

The human fetal osteoblast cell line hFOB 1.19 was obtained from the American Type Culture Collection (ATCC, catalog no. CRL-11372). The cells are attachment dependent and are propagated in the growth medium specified by ATCC: a 1:1 mixture of Ham's F12 medium (Gibco catalog no. 21700, powder) and Dulbecco's modified Eagle's medium (Gibco catalog no. 13000, powder) with 2.5 mM L-glutamine and supplemented with 2.44 g/liter sodium bicarbonate, 0.3 g/liter neomycin G418, and 10% fetal bovine serum. These cells have been transfected with a reporter gene for the expression of the enzyme alkaline phosphatase, hence G418 is needed for selection pressure. As explained in the Product Information Sheet from ATCC, when these cells are grown at the permissive temperature of 34°C, they exhibit rapid cell division. However, little or no cell division occurs at a restrictive temperature of 39.5°C, rather, the cells differentiate and a more mature osteoblast phenotype is produced. Because undifferentiated, rapid cell growth was wanted, these osteoblasts were grown in an incubator at 34°C in an atmosphere of 5% CO₂. ATCC reports a doubling time of 36 hours for these cells at 33.5°C.

Upon thawing, the cells were transferred to a 15 ml centrifuge tube containing 10 ml of complete medium, centrifuged at ~800 rpm for 3 minutes, resuspended in 8 ml of complete medium, and transferred to 75 cm² tissue culture flask. Osteoblasts were subcultured upon reaching confluence in the flask by removing the culture medium from the flask, washing the cells once with PBS (phosphate buffered saline) containing 0.2 g/liter EDTA3Na (ethylene diamine tetracetic acid), then detaching the cells by incubating them in the presence of 0.25% trypsin-EDTA for 4 minutes at 34°C. The trypsin was inactivated by the addition of complete medium and the osteoblasts were diluted 1:5 with fresh medium in a new flask.

The experiment to assess growth of the osteoblasts on the calcium phosphate microspheres was done in a 250 ml spinner flask, which had been silicized to inhibit the attachment of cells to the glass walls of the spinner flask. A volume of 20 ml of calcium phosphate hollow microspheres (specific gravity of 1.05 grams/cc- 2 mm diameter- H₂O impervious surface) was placed in the spinner flask with about 30 ml of cell culture grade water and sterilized by autoclaving for 30 minutes. After the flask cooled, the water was removed and the calcium phosphate microspheres were washed twice with 25 ml of complete medium. Inoculum for the experiment was grown in a 162 cm² tissue culture flask. When the osteoblasts reached confluence, the cells were detached by trypsinization, diluted in 39 ml of complete growth medium and transferred to the sterile spinner flask. A sample of the inoculum was used for counting the cells to determine the seed concentration. It was determined that 1.076×10^7 cells were added to the spinner. The spinner was placed in the incubator without agitation to allow the cells to attach. After about 12 hours, the agitation was started at 20 rpm in order to provide better oxygen transfer into the medium. Glucose and lactic acid were assayed daily as an indirect measurement of cell growth. The growth medium was replaced every four days or whenever the glucose concentration fell below 1.5 g/liter. Multiple layers of cells were demonstrated to grow under these conditions as shown by scanning electron microscope micrographs.

While various embodiments of the present invention have been described in detail, it is apparent that modifications and adaptations of those embodiments will occur to those skilled in the art. It is to be expressly understood, however, that such modifications and adaptations are within the scope of the present invention.

Claims:

1. A composition comprising hollow calcium-containing microstructures and a bone mixture.

2. The composition of claim 1, wherein the microstructures are about 0.5 mm to about 6 mm in diameter.

3. The composition of claim 1, wherein the bone mixture comprises bone tissues or bone by-products.

4. The composition of claim 1, wherein the bone mixture is from about 5% to about 95% by volume of the composition.

5. The composition of claim 1, wherein the bone mixture is from about 50% to about 75% by volume of the composition.

6. The composition of claim 1, wherein the calcium-containing microstructures comprise hydroxylapatite, tribasic calcium phosphate, dicalcium phosphate, tetracalcium phosphate, calcium carbonate, calcium oxide, glass-containing calcium phosphate, or a mixture thereof.

7. The composition of claim 1, wherein the composition further comprises a bonding agent.

8. The composition of claim 7, wherein the bonding agent is a polymer.

9. The composition of claim 8, wherein the polymer is polylactic acid, polyglycolic acid, polycaprolactone, poly α -hydroxy esters, polyphosphazenes, polyanhydrides, and polypropylene fumarate.

10. The composition of claim 7, wherein the bonding agent is a calcium-containing cement.

11. The composition of claim 10, wherein the calcium-containing cement is from about 5% to about 75% by volume of the composition.

12. The composition of claim 10, wherein the calcium-containing cement is from about 10% to about 50% by volume of the composition.

13. The composition of claim 10, wherein the calcium-containing cement is calcium phosphate, calcium sulfate or a mixture thereof.

14. The composition of claim 10, wherein the calcium-containing cement comprises calcium sulfate.

15. A calcium composition comprising porous or hollow microstructures or particulate containing nitric oxide or a nitric oxide forming compound.

16. The composition of claim 15, wherein the microstructures comprise hydroxylapatite, tribasic calcium phosphate, tetracalcium phosphate, dicalcium phosphate, calcium carbonate, calcium oxide, glass-containing calcium phosphate, or a mixture thereof.

17. The composition of claim 15, wherein the microstructures comprise glass.

18. The composition of claim 17, wherein the glass contains calcium phosphate.

19. The composition of claim 15, wherein the microstructures comprise a polymer.

20. The composition of claim 15, wherein nitric oxide is contained within the composition as a gas, in a liquid or a mixture thereof.

21. The composition of claim 15, wherein the microstructures are about 250 μm to about 6 mm in diameter.

22. The composition of claim 15, wherein said composition further comprises a nitric oxide inhibitor or activator.

23. The composition of claim 15, wherein the nitric oxide forming compound is nitric oxide synthase or L-arginine.

24. The composition of claim 15, wherein the porous or hollow microstructures contain nitric oxide or nitric oxide forming compound, and wherein the nitric oxide forming compound is nitric oxide synthase.

25. The composition of claim 15, wherein the composition is coated with a bioresorbable polymer.

26. The composition of claim 25, wherein the polymer is polylactic acid, polyglycolic acid, polycaprolactone, poly α -hydroxy esters, polyphosphazenes, polyanhydrides, or polypropylene fumarate.

27. A method of delivering nitric oxide to a desired site, comprising contacting the site with the composition of claim 15.

28. The method of claim 27, wherein the composition is administered to a patient in need of nitric oxide at the desired site.

29. The method of claim 27, wherein an effective amount of nitric oxide or the nitric oxide forming compound is contained within the microstructures for use as a growth factor.

30. The method of claim 27, wherein a cytotoxic effective amount of nitric oxide or the nitric oxide forming compound is contained within the microstructures to treat a pathogen, a tumor, or an invading organism.

31. The method of claim 27, wherein an effective amount of nitric oxide or nitric oxide forming compound is contained within the microstructures to improve immune system regulation, vascular tone or neural signaling in a patient in need thereof.

32. The method of claim 27, wherein an inhibitor or activator of nitric oxide is further administered to the patient to regulate the activity of nitric oxide.

33. A method of making a calcium-containing substrate, comprising the steps of:

- (a) obtaining a composition comprising calcium phosphate;
- (b) contouring said composition into a desired shape to form a contoured composition;
- (c) culturing cells or tissues onto the contoured composition to form the calcium-containing substrate.

34. The method of claim 33, further comprising the steps of:

- (d) removing the cultured cells or tissues from the contoured composition; and
- (e) transplanting the cultured cells or tissues into a patient in need thereof.

35. The method of claim 33, further comprising the step of transplanting the calcium-containing substrate into a patient in need thereof.

36. The method according to claims 33, 34 or 35, wherein calcium phosphate is hydroxylapatite, tribasic calcium phosphate, dicalcium phosphate, tetracalcium phosphate, calcium carbonate, calcium oxide, glass-containing calcium phosphate, or a mixture thereof.

37. The method according to claims 33, 34 or 35, wherein said composition is contoured by tape casting, glass forming, or cad-cam processing.

38. The method of claim 33, wherein said contoured composition is fired prior to step (c).

39. The method according to claims 33, 34 or 35, wherein the desired shape is obtained from a patient by computer aided tomography to form a mold of the desired shape.

40. The method of claim 33, further comprising the step of administering a decalcifying agent to dissolve the calcium after step (c).

41. The method of claim 33, wherein the cells or tissues can be cultured on both sides of the contoured composition.

42. The method of claim 41, wherein one cell or tissue type is cultured on one side of the contoured composition and a second cell or tissue type is cultured on a second side of the contoured composition.

43. The method according to claims 37 or 39, wherein the contoured composition is formed by layers of cast tape.



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[54] SOFT TISSUE AUGMENTATION MATERIAL

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[62] Division of application No. 08/159,071, Nov. 29, 1993, which is a continuation of application No. 07/999,411, Jan. 21, 1993, abandoned, which is a continuation-in-part of application No. 07/833,874, Feb. 11, 1992, abandoned.

[51] Int. Cl.⁶ A61F 2/02[52] U.S. Cl. 623/11; 623/8; 623/66;
623/16; 523/113; 523/115; 424/423[58] Field of Search 523/113, 115;
424/422, 423, 484, 486, 487, 488, 489,
499; 524/415, 417; 604/56; 623/11, 16,
18, 7, 8, 9, 10, 12

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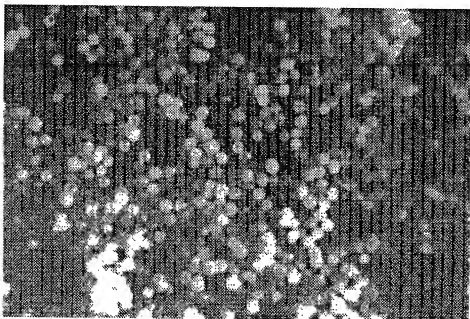
Primary Examiner—John G. Weiss

Assistant Examiner—Francis K. Cuddihy
 Attorney, Agent, or Firm—Theodore R. Furman, Jr.; John M. Kilcoyne; Stuart E. Krieger

[57] ABSTRACT

A permanent, biocompatible material for soft tissue augmentation. The biocompatible material comprises a matrix of smooth, round, finely divided, substantially spherical particles of a biocompatible ceramic material, close to or in contact with each other, which provide a scaffold or lattice for autogenous, three dimensional, randomly oriented, non-scar soft tissue growth at the augmentation site. The augmentation material can be homogeneously suspended in a biocompatible, resorbable lubricious gel carrier comprising a polysaccharide. This serves to improve the delivery of the augmentation material by injection to the tissue site where augmentation is desired. The augmentation material is especially suitable for urethral sphincter augmentation, for treatment of incontinence, for filling soft tissue voids, for creating soft tissue blebs, for the treatment of unilateral vocal cord paralysis, and for mammary implants. It can be injected intradermally, subcutaneously or can be implanted.

48 Claims, 2 Drawing Sheets



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Patent and Trademark Office: U.S. DEPARTMENT OF COMMERCE

DO/PTO Rev. 6/95 DECLARATION FOR UTILITY OR DESIGN PATENT APPLICATION <input type="checkbox"/> Declaration Submitted with Initial Filing OR <input checked="" type="checkbox"/> Declaration Submitted after Initial Filing	U.S. Department of Commerce Patent and Trademark Office	Attorney Docket Number	4141-2-PUS
		First Named Inventor	STARLING, L. Brian
	COMPLETE IF KNOWN		
	Application Number	10/030,578	
	Filing Date		
	Group Art Unit		
		Examiner Name	

As below named inventor, I hereby declare that::

My residence, post office address, and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed for which a patent is sought on the invention entitled:

"CALCIUM-CONTAINING STRUCTURES AND METHODS OF MAKING AND USING THE SAME"

(Title of the Invention)

the specification of which

☐ is attached hereto

OR

☒ was filed on
(MM/DD/YYYY)

07 July 2000

as United States Application Number or PCT International

Application Number

PCT/US00/18712

and was amended on
(MM/DD/YYYY)

(if applicable)

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment specifically referred above.

I acknowledge the duty to disclose information which is material to patentability as defined in Title 37 Code of Federal Regulations, § 1.56.

I hereby claim foreign priority benefits under Title 35, United States Code § 119 (a)-(d) or § 365(b) of any foreign application(s) for patent or inventor's certificate, or §365(a) of any PCT international application which designated at least one country other than the United States of America, listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate, or of any Pct international application having a filing date before that of the application on which priority is claimed.

Prior Foreign Application Number(s)	Country	Foreign Filing Date (MM/DD/YYYY)	Priority Not Claimed	Certified Copy Attached?	
				Yes	No
			<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
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☐ Additional foreign application numbers are listed on a supplemental priority sheet attached hereto.

I hereby claim the benefit under Title 35, United States Code § 119(e) of any United States provisional application(s) listed below

Application Number(s)	Filing Date (MM/DD/YYYY)		
60/143,333	July 8, 1999	<input type="checkbox"/>	Additional provisional application numbers are listed on a supplemental priority sheet attached hereto.

DECLARATION

Page 2

I hereby claim the benefit under Title 35, United States Code § 120 of any United States application(s), or § 365(c) of any PCT International application designating the United States of America, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States of PCT International application in the manner provided by the first paragraph of Title 35, United States Code § 112.1 acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations § 1.56 which became available between the filing date of the prior application and the national or PCT International filing date of this application.

U.S. Parent Application Number	PCT Parent Number	Parent Filing Date (MM/DD/YYYY)	Parent Patent Number (if applicable)

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As a named inventor, I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith:

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BLAKELY, TODD P.	34,328	SWARTZ, DOUGLAS W.	37,739
CONNELL, GARY J.	32,020	KUGLER, BRUCE A.	38,942
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I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true, and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Name of Sole or First Inventor: ☐ A petition has been filed for this unsigned inventor

Given Name **L. Brian** Middle Initial Family Name **Starling** Suffix **e.g. Jr.**

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ADDITIONAL INVENTOR(S) Supplemental Sheet

Name of Additional Joint Inventor, if any:

☐ A petition has been filed for this unsigned inventor

Given Name	James	Middle Initial	E.	Family Name	Stephan	Suffix e.g. Jr.	
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Inventor's Signature

James E Stephan

Date

4-2-02 April 2, '02

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☐ A petition has been filed for this unsigned inventor

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Inventor's Signature

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Given Name		Middle Initial		Family Name		Suffix e.g. Jr.	
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Inventor's Signature

Date

Residence City		State		Country		Citizenship	
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City		State		Zip		Country		Applicant Authority	
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[] Further applicants and/or (further) inventors are indicated on another continuation sheet

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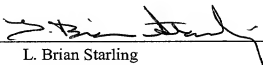
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Supplemental Sheet

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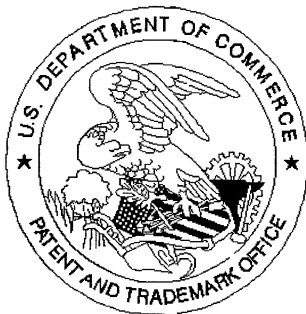
On behalf of CaP Biotechnology, Inc., a Colorado corporation having a principal place of business at 15400 W. 44th Avenue, Unit C, Golden, Colorado 80403 being the assignee of and owning all right, title and interest in the invention entitled "CALCIUM-CONTAINING STRUCTURES AND METHODS OF MAKING AND USING THE SAME", for which application for Letters Patent of the United States has been made by L. Brian Starling, James E. Stephan, , said application having been filed on January 8, 2002, receiving Serial No. 10/030,578, and further identified as Attorney File No. 4141-2-PUS, I, hereby appoint David F. Zinger, Registration No. 29,127; Craig C. Groseth, Registration No. 31,713; Sabrina C. Stavish, Registration No. 33,374; Todd P. Blakely, Registration No. 31,328; Lewis D. Hansen, Registration No. 35,536; Joseph E. Kovarik, Registration No. 33,005; Gary J. Connell, Registration No. 32,020; Douglas W. Swartz, Registration No. 37,739; Bruce A. Kugler, Registration No. 38,942; Robert R. Brunelli, Registration No. 39,617; Brent P. Johnson, Registration No. 38,031; Dana Hartje Cardwell, Registration No. 40,638; Angela K. Dallas, Registration No. 42,460; Benjamin B. Lieb, Registration No. 42,801; Bradley M. Knepper, Registration No. 44,189; Miriam Drickman Trudell, Registration No. 42,499; Dennis J. Dupray, Registration No. 46,299; Kenneth C. Winterton, Registration No. 48,040; Robert D. Traver, Registration No. 47,999; Mark L. Yaskanin, Registration No. 45,246; and Mollybeth R. Kocalski, Registration No. 42,754, of SHERIDAN ROSS P.C., 1560 Broadway, Suite 1200, Denver, Colorado 80202-5141, telephone number (303) 863-9700, as attorneys and agents for CaP Biotechnology, Inc. with full powers of substitution, association and revocation to prosecute the application and related U.S. and foreign applications and to transact all business in the United States Patent and Trademark Office and all foreign and international patent offices connected therewith.

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By: 
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Vice President

Date: April 2, 2002

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